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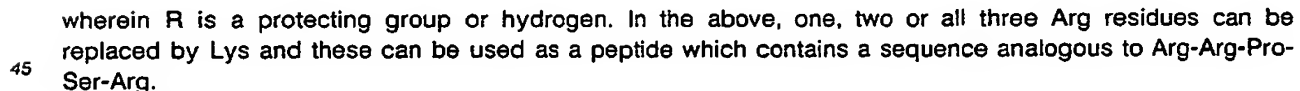
(54) **Kit or reagent for assaying the cellular integrity of blood platelets and pharmaceutical compositions containing a PKA inhibitor.**

(57) There is provided a kit or reagent for assaying the cellular integrity (intactness) of blood platelets comprising means for determining the quantity of PKA released by a number of platelets into a certain volume of plasma. The assay is based on determining cAMP-dependent phosphorylation of vitronectin (protein S) in plasma using radioactively labelled [ $\gamma$ -<sup>32</sup>P]ATP as phosphate donor. The assay can be based on the specific inhibition of the phosphorylation in blood fluid by protein kinase inhibitors. The invention further relates to pharmaceutical compositions for the treatment of pathological conditions associated with impaired platelet function in humans, which comprises administering to a person in need thereof a physiologically active quantity of a PKA inhibitor adapted to decrease or prevent vitronectin phosphorylation. The inhibitor can be a synthetic or genetically engineered peptide.

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The invention relates to a kit or reagent for assaying the cellular integrity (intactness) of blood platelets which comprises means for determining the quantity of cAMP-dependent protein kinase (PKA) released by a number of stimulated platelets into a certain volume of plasma. More specifically it relates to a kit or reagent wherein cAMP-dependent phosphorylation of vitronectin (protein S) is determined in plasma using 5 radioactively labelled ATP as phosphate donor, comprising means for determining the radioactivity of the PKA-phosphorylated vitronectin.

A specific embodiment relates to a kit or reagent for assaying the biological functionality of isolated blood platelets which comprises means for determining their ability to release PKA when stimulated with a physiological platelet agonist (thrombin, collagen, ADP etc.) wherein the determination of the released PKA is effected as described above. Furthermore the invention relates to a pharmaceutical composition for the treatment of pathological conditions associated with impaired platelet function in humans, which comprises administering to such a person in need thereof a physiologically active quantity of a PKA inhibitor adapted to decrease or prevent vitronectin phosphorylation. Preferably the inhibitor is a synthetic or genetically engineered peptide containing any of the sequences



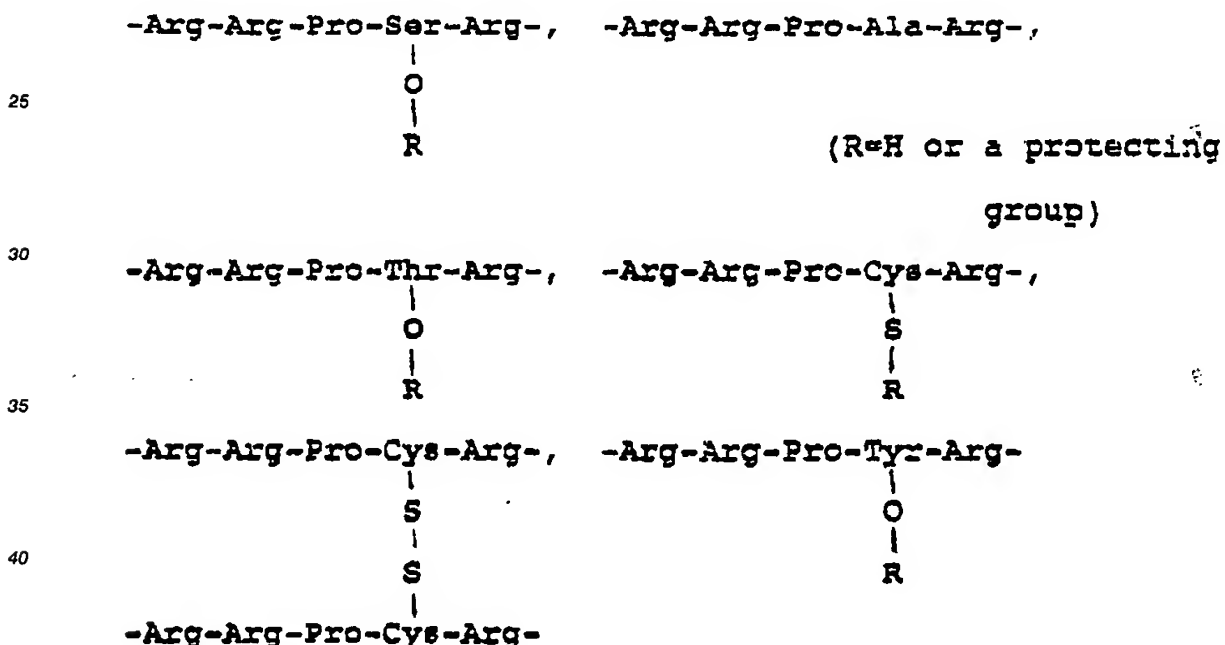
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they release PKA into the blood fluid, and that in human plasma there is a specific target substrate (p75) for this kinase (5). This target substrate is shown to be vitronectin, a protein which has been implicated in cell adhesion, complement function and blood clotting (6-11).

The invention relates to a kit or reagent for assaying the cellular integrity (intactness) of blood platelets which comprises means for determining the quantity of PKA released by a number of stimulated platelets into a certain volume of plasma. More specifically it relates to a kit or reagent wherein cAMP-dependent phosphorylation of vitronectin (protein S) is determined in plasma using radioactively labelled ATP as phosphate donor, comprising means for determining the radioactivity of the PKA-phosphorylated vitronectin.

According to a preferred embodiment the specific inhibition of the phosphorylation in blood fluid by protein kinase inhibitors is determined. There is also provided a radioimmunoassay for assessing the cellular integrity of blood platelets, which comprises means for monitoring the release of PKA in plasma by means of anti-PKA, anti-phosphvitronectin or anti-vitronectin antibodies.

A specific embodiment relates to a kit or reagent for assaying the biological functionality of isolated blood platelets which comprises means for determining their ability to release PKA when stimulated with a physiological platelet agonist (thrombin, collagen, ADP etc.) wherein the determination of the released PKA is effected as described above. Furthermore the invention relates to a pharmaceutical composition for the treatment of pathological conditions associated with impaired platelet function in humans, which comprises administering to such a person in need thereof a physiologically active quantity of a PKA inhibitor adapted to decrease or prevent vitronectin phosphorylation. Preferably the inhibitor is a synthetic or genetically engineered peptide containing any of the sequences



45 wherein R is a protecting group or hydrogen. In the above, one, two or all three of the Arg residues can be replaced by Lys and these can be used as a peptide which contains a sequence analogous to Arg-Arg-Pro-Ser-Arg.

Furthermore the invention relates to a method for determining the integrity and or biological functionality of blood platelets which comprises determining the quantity of PKA released by such platelets into plasma and evaluating the quantity released. It further relates to a method for the determination of the cellular integrity and/or biological functionality of blood platelets based on the determination of cAMP dependent phosphorylation of vitronectin in plasma and/or the inhibition of such phosphorylation with radioactively labelled [ $\gamma$ - $^{32}$ P] ATP as phosphate donor, and determining the degree of phosphorylation, and/or its prevention which is indicative of the quantity of PKA released by the platelets into the plasma.

55 Activation of freshly isolated human blood platelets with physiological stimulants (e.g. thrombin) caused them to release a cAMP-dependent protein kinase which specifically phosphorylates one plasma protein (M, 75000)(5). This protein was immunochemically and biochemically identified as vitronectin (also known as S protein), which was previously implicated in blood clotting, complement function and cell adhesion. Our



preparation used. These bands could be reproduced (lane E) by incubation of the same PKI preparation with the catalytic subunit of PKA and [ $\gamma$ - $^{32}$ P]ATP. Lane F: Samples of the same sera (5  $\mu$ l) were similarly electrophoresed and stained with 0.25 % Coomassie blue.

Fig. 2 Portions (45  $\mu$ l) of fresh blood (obtained from volunteers as described in Example I) were withdrawn into polypropylene tubes containing 5 ml of trisodium citrate (3.8 % w/v). Protein phosphorylation was carried out in the presence of added cAMP ( $10^{-5}$  M) and PKI (0.1 mg/ml), as indicated by the dots at the bottom of the figure. Analysis by gel electrophoresis (polyacrylamide 5-15 %), and autoradiography were carried out as described previously (3). Panel A: platelet-free plasma (endogenous phosphorylation on samples of 5  $\mu$ l). Panel B: Platelet-containing plasma ( $5 \times 10^8$  platelets/ml) stimulated with thrombin (1 U/ml). The suspension was incubated (30 min at 22 °C), centrifuged (10 min at 10000 rpm) and aliquots (5  $\mu$ l) were assayed for endogenous phosphorylation. Panel C: Gel-filtered platelets stimulated with thrombin. A preparation of gel-filtered platelets ( $5 \times 10^8$  platelets/ml) was stimulated with thrombin (1 U/ml), incubated (30 min at 22 °C), centrifuged (10 min. at 5000 rpm) and aliquots (20  $\mu$ l) of the supernatant were assayed for endogenous phosphorylation. Panel D: Reconstitution of endogenous phosphorylation by combining the supernatant (20  $\mu$ l) of thrombin-stimulated platelets (C) with 5  $\mu$ l of platelet-free plasma (A). The mixture was subjected to endogenous phosphorylation. Panel E: Simulation of the endogenous phosphorylation occurring in thrombin-stimulated platelet-containing plasma by addition of pure catalytic subunit of rabbit muscle PKA (final concentration 5  $\mu$ g/ml) to platelet-free plasma (A). Samples of 5  $\mu$ l were subjected to phosphorylation and analysis as described in the legend to Fig. 1. For the low  $M_r$  (<35000) band seen in lanes containing PKI see legend to Fig. 1.

Fig. 3 A sample (250  $\mu$ l) of human plasma was applied on a column (6.3 x 1 cm) of DEAE Sephadex equilibrated and run (22 °C) with a buffer composed of 2-(N-morpholino)ethanesulfonic acid (5mM), EDTA (1 mM) and NaCl (9mM), pH 6.5. A linear NaCl gradient (9 to 250 mM NaCl in the equilibration buffer, total volume 50 ml) was applied, followed by a 10 ml final step of 500 mM NaCl in the same buffer. Fractions (1 ml) were collected and the following parameters were followed: protein concentration (●), conductivity (□), [ $^{32}$ P] incorporation (○). The phosphorylation by PKA was assayed on aliquots (20  $\mu$ l) of the indicated fractions. The reaction mixtures contained the following constituents at the indicated final concentrations: pure catalytic subunit of PKA from rabbit muscle (5  $\mu$ g/ml),  $Mg(CH_3COO)_2$  (10 mM), [ $\gamma$ - $^{32}$ P]ATP 10  $\mu$ M, 50 Ci/mmol). Phosphorylation was allowed to proceed for 10 min at 30 °C, arrested as described in the legend to Fig. 1, and subjected to gel electrophoresis (polyacrylamide 5-15 %) and autoradiography (inset). The lanes of the dry gels were cut out, and their radioactivity was counted in a scintillation counter using a toluene-based scintillant.

Fig. 4. Samples (20  $\mu$ l) of the p75 peak fraction (number 58) in the experiment depicted in Fig. 3 were phosphorylated as described in the legend of that figure, bringing the total volume of the sample to 50  $\mu$ l. Phosphorylation was arrested by addition of 25  $\mu$ l of buffer I composed of NaP (150 mM),  $-Na_4P_2O_7$  (15 mM), EDTA (45 mM),  $Na_2VO_4$  (6 mM), ATP (9 mM), Hepes (150 mM), pH 7.5. To each aliquot (75  $\mu$ l) of the arrested reaction mixture, a suspension (5  $\mu$ l) of Protein A Sepharose (50 % v/v in buffer I) was added (to remove any constituent that might react non-specifically with Protein A Sepharose). After an incubation of 15 min at 4 °C, the samples were centrifuged (2 min at 12000 rpm), the supernatants were collected and incubated with 20  $\mu$ l of one of the solutions of goat antibodies against vitronectin ( $\alpha$ V), fibrinogen ( $\alpha$ F) and plasminogen ( $\alpha$ P) dissolved in buffer I diluted 1:3 (final concentrations 5mg/ml). After an incubation of 3 h at 4 °C Protein A Sepharose (20  $\mu$ l of 50 % v/v in buffer I) was added and the incubation was continued for an additional hour at 4 °C. The samples were centrifuged (2 min at 12000 rpm) and the supernatants (marked S in the figure) were boiled (5 min at 98 °C in the presence of sodium dodecylsulfate (2 %) and 2-mercaptoethanol (0.15 M). The pellets (marked P in the figure) containing the immuno-complexes were washed twice with a solution composed of Hepes (50 mM), pH 7.5: Triton X-100 (1 % v/v); sodium dodecylsulfate (1 % w/v) and then once more with a solution composed of Hepes (50 mM), pH 7.5; Triton X-100 (1 % v/v); NaCl (150 mM). They were then dissolved by addition of 50  $\mu$ l of a buffer solution containing sodium dodecylsulfate (2 % w/v) and 2-mercaptoethanol (0.75 M) and boiled (5 min at 98 °C). The supernatants (S) and pellets (P) were subjected to polyacrylamide gel electrophoresis (polyacrylamide 5-15 %) and autoradiography.

Fig. 5 Inhibition of vitronectin phosphorylation by PKA in the presence of the indicated concentrations of a peptide identical in sequence to the site phosphorylated in vitronectin.

Fig. 6 Vitronectin (2.7  $\mu$ M) was incubated for 2 h at 22 °C with C (1  $\mu$ g/ml) in the presence of  $Mg(CH_3COO)_2$  (10 mM), [ $\gamma$ - $^{32}$ P]ATP 50  $\mu$ M, 0.5 Ci/mmol), (50 Mes at the indicated pH), and a 3-fold molar excess of heparin over vitronectin (where indicated). The reaction was terminated by boiling in Laemli's sample buffer and the extent of phosphorylation was analysed by polyacrylamide gel electrophoresis and autoradiography.

The following examples illustrate the invention.

#### Example I

Fresh human blood obtained by venipuncture of healthy individuals, who had fasted overnight and who had ingested no drugs for at least two weeks prior to blood withdrawal, was allowed to clot in a glass tube and the resulting serum was subjected to endogenous phosphorylation with [ $\gamma$ - $^{32}$ P]ATP and  $Mg^{++}$ . One protein band ( $M_r$  75000) became phosphorylated (Fig. 1, lane A). This phosphorylation was enhanced by added cAMP (Fig. 1, lane B) and prevented by the Walsh-Krebs (13) specific PKA inhibitor (PKI) (Fig. 1, lanes C and D) indicating that it was catalyzed by PKA.

As seen in Fig. 1, the endogenous phosphorylation in serum was specific: one protein was singled out from the large repertoire of serum proteins (Fig. 1, compare lanes A and B with lane E) and became phosphorylated in a cAMP dependent process. The protein phosphorylated in human blood had a molecular weight of 75000 (p75), and was shown to be vitronectin (v.i) (5).

#### Example II

Fresh human blood (see Example I) was used to prepare platelet-containing plasma, platelet-free plasma and gel-filtered ("washed") platelets, taking measures to avoid platelet breakage. Each of these preparations was subjected to phosphorylation with [ $\gamma$ - $^{32}$ P] ATP and  $Mg^{++}$ , in the presence of added cAMP, with or without PKI. As seen in Fig. 2, panel A, no phosphorylation of p75 vitronectin occurred in platelet-free plasma alone. However, when platelet-containing plasma was stimulated with thrombin and the resulting white clot was spun down, p75 was subsequently phosphorylated in the supernatant and this phosphorylation was prevented by PKI (see Fig. 2, panel B). On the other hand, when intact, gel-filtered platelets were stimulated with thrombin, then spun down, and their supernatant subjected to endogenous phosphorylation under the same conditions, no evidence for the presence of p75 could be obtained (Fig. 2, panel C). Interestingly, though neither this supernatant alone nor the platelet-free plasma alone exhibited phosphorylation of p75, phosphorylation of p75 could be reproduced in the presence of added cAMP, and prevented by PKI (Fig. 2, panel D). Finally, the specific phosphorylation of p75 could be simulated with pure catalytic subunit of PKA (from rabbit muscle (14) added to platelet-free plasma (Fig. 2, panel E). As expected, also this phosphorylation was prevented by PKI.

#### Example III

The specificity of plasma vitronectin phosphorylation by PKA is illustrated in tracing p75 during its purification, using the catalytic subunit of PKA as a reagent. When platelet-free plasma was subjected to ion exchange chromatography on DEAE-Sephadex with a linear salt gradient, most of the proteins were eluted between 0 and 250 mM NaCl. Upon raising the salt concentration with NaCl, a small protein peak emerged. Using the PKA assay mentioned above, this peak was shown to contain all of p75 (purification >50 fold). It should be noted that a very similar elution pattern was also obtained with human serum (sometimes with a minor  $M_w$  65000 band (p65) ) even when the phosphorylation with PKA was carried out prior to chromatography, i.e. in the presence of all the other serum proteins.

#### Example IV

Using antibodies against various human serum constituents, it was possible to immunochemically identify p75 as vitronectin (5) (also known as serum spreading factor or complement S protein. The p75 purified and [ $P^{32}$ ] labelled was quantitatively precipitated with goat antibodies against human vitronectin ( $\alpha V$ ) but not with goat antibodies against human fibrinogen ( $\alpha F$ ) or plasminogen ( $\alpha P$ ) (Fig. 4). Vitronectin (p75 with a minor p65 band) also precipitated from whole serum with  $\alpha V$  (not shown).

The identification of p75 as vitronectin is also in agreement with its reported molecular weight, with its co-precipitation by  $\alpha V$  with p65 (possibly another biologically active form of p75 and with its behaviour on ion exchange columns (6-8).

Example V

Peptides containing the sequence Arg-Arg-Pro-X-Arg (X = Ser, Thr, Ala, Cys, Tyr) inhibit the phosphorylation of vitronectin by PKA. Apart from PKI (see Figs 1 and 2) a few other peptides were found to do so. For example, the octapeptide which had a sequence identical to that found at the site of phosphorylation of vitronectin (Ser 378), namely Ser-Arg-Arg-Pro-Ser-Arg-Ala-Thr, (X = Ser) was phosphorylated by PKA and thus competed with (inhibited) the phosphorylation of vitronectin (Fig. 5) with a Ki concentration of 30  $\mu$ M.

Example VI

The phosphorylation of vitronectin (p75 and mainly the p65 form) is affected by pH and by the presence of heparin in the reaction mixture (Fig. 6).

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Claims

1. A kit or reagent for assaying the cellular integrity (intactness) of blood platelets which comprises means for determining the quantity of PKA released by a number of platelets into a certain volume of plasma.
2. A kit or reagent according to claim 1, wherein the cAMP-dependent phosphorylation of vitronectin (protein S) is determined in plasma using radioactively labelled [ $\gamma^{32}$ P]ATP as phosphate donor, optionally in the presence of heparin, comprising means for determining the radioactivity of the PKA-phosphorylated vitronectin.
3. A kit or reagent according to claim 1 or 2, wherein the specific inhibition of the phosphorylation in blood fluid by protein kinase inhibitors is used.
4. A kit or reagent according to claim 1 for carrying out a radioimmunoassay, which comprises means for monitoring the release of PKA in plasma by means of anti-PKA, anti-phosphovitronectin or anti-vitronectin antibodies.
5. A kit or reagent according to any one of claims 1 to 4, wherein the substrate of PKA is purified vitronectin, an animal protein with at least one of the activities of vitronectin, or a biologically active fragment of vitronectin.
6. A kit or reagent according to claim 5, wherein the substrate of PKA is a genetically engineered protein which exhibits at least one vitronectin-like activity.
7. A kit or reagent according to claim 6, wherein the substrate of PKA is the heparin binding domain of vitronectin.
8. A kit or reagent according to claim 6, wherein the substrate of PKA is a synthetic or genetically

engineered peptide which contains the sequence Arg-Arg-Pro-X-Arg where X is Ser or Thr.

9. A kit or reagent according to claim 8, wherein one, two or all three of the Arg residues is replaced by Lys.

10. A kit or reagent according to claim 8, wherein the PKA substrate is a peptide containing the sequence Arg-Arg-Pro-Ser-Arg.

11. A kit or reagent according to any one of the claims 1 to 10 for assaying the biological functionality of isolated blood platelets which comprises means for determining their ability to release PKA when stimulated with a physiological platelet agonist, preferably with thrombin, collagen or ADP.

12. PKA inhibitor adapted to decrease or prevent vitronectin phosphorylation.

13. PKA inhibitor according to claim 12, which is a synthetic or genetically engineered peptide containing any of the sequences

-Arg-Arg-Pro-Ser-Arg-, -Arg-Arg-Pro-Ala-Arg-,



-Arg-Arg-Pro-Thr-Arg-, -Arg-Arg-Pro-Cys-Arg-,



-Arg-Arg-Pro-Cys-Arg-, -Arg-Arg-Pro-Tyr-Arg-



-Arg-Arg-Pro-Cys-Arg-

wherein R is a protecting group or a hydrogen atom.

14. PKA inhibitor according to claim 13, wherein one, two or all three of the Arg residues is replaced by Lys.

15. A pharmaceutical composition containing a PKA inhibitor according to any one of claims 12 to 14 and optionally in addition a pharmaceutically acceptable carrier or diluent.

16. A pharmaceutical composition according to claim 15 for the treatment of pathological conditions associated with impaired platelet function.

17. A composition according to claim 15 or 16 additionally containing an effective quantity of heparin, heparan sulfate or an equivalent thereof.

18. A method for determining the integrity and/or biological functionality of blood platelets which comprises determining the quantity of PKA released by such platelets into plasma and evaluating the quantity released.

19. A method for the determination of the cellular integrity and/or biological functionality of blood platelets based on the determination of cAMP dependent phosphorylation of vitronectin in plasma and/or the inhibition of such phosphorylation with radioactively labelled ATP as phosphate donor, and determining the degree of phosphorylation, and/or its prevention which is indicative of the quantity of PKA released by the platelets into the plasma.



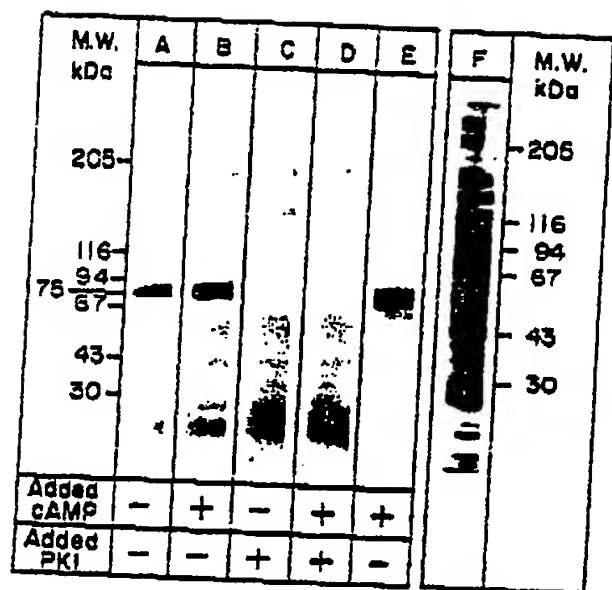


Fig. 1

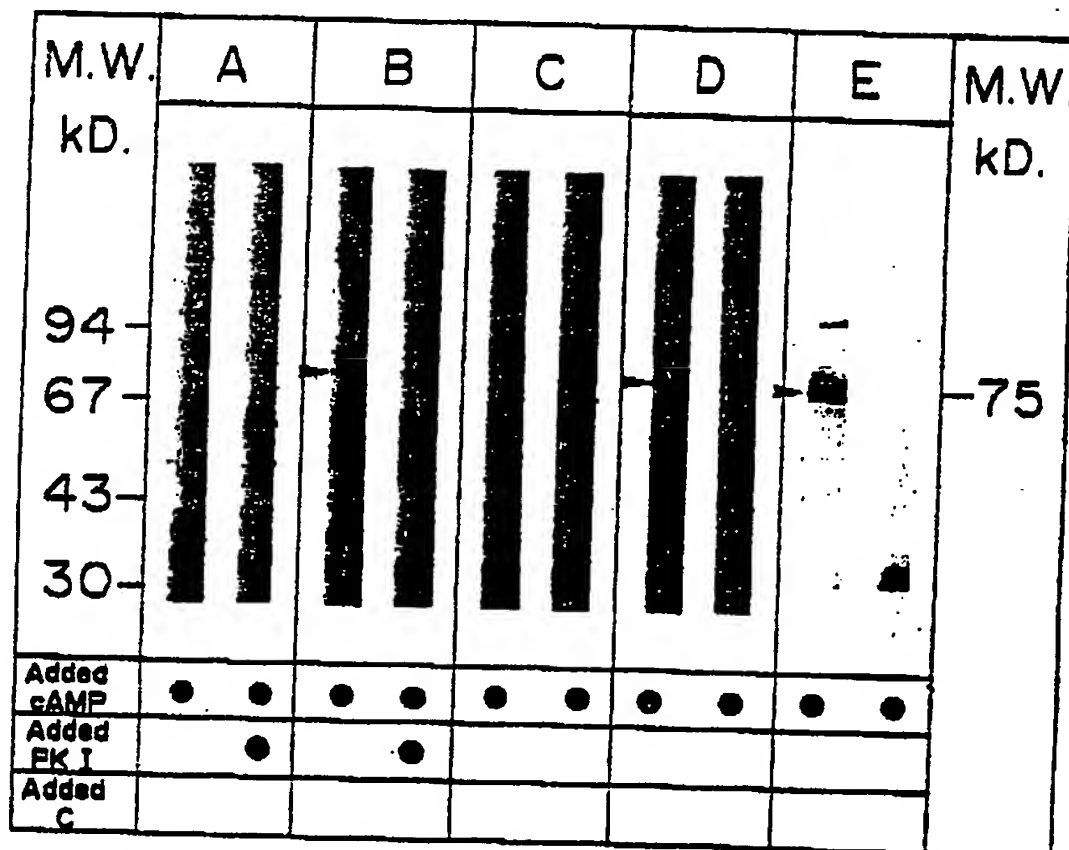


Fig. 2

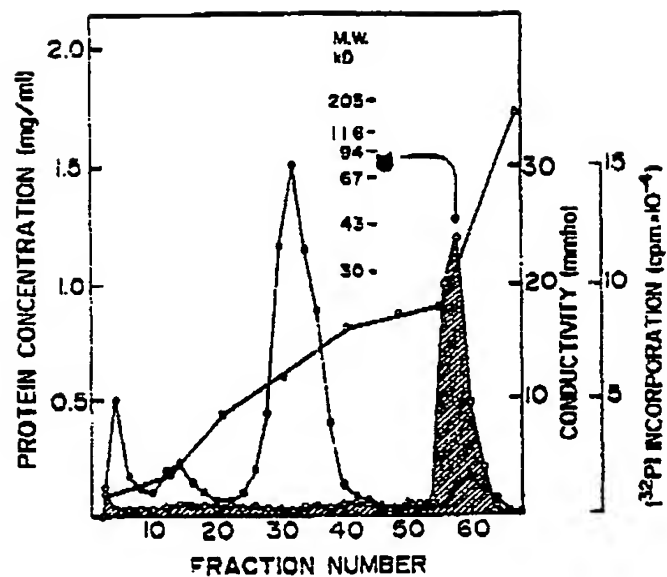


Fig. 3

M.W. kD.	$\alpha\text{V}$	$\alpha\text{F}$	$\alpha\text{P}$
94			
67			
43			
30			
	S P	S P	S P

Fig. 4

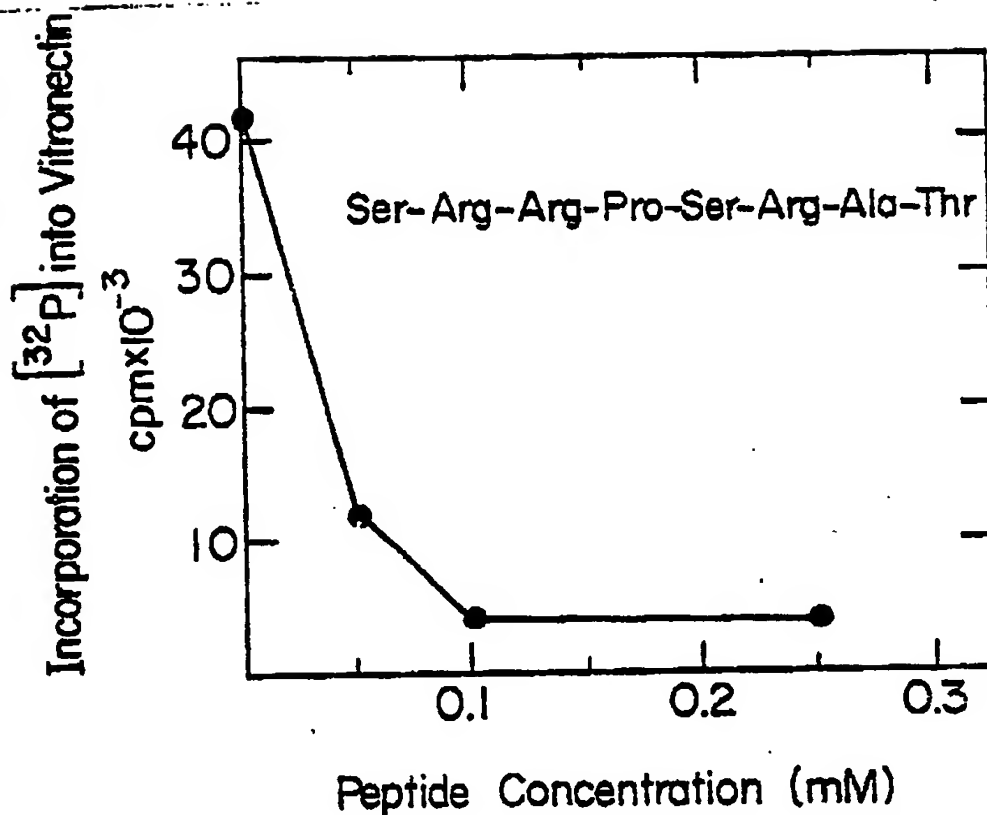


Fig. 5

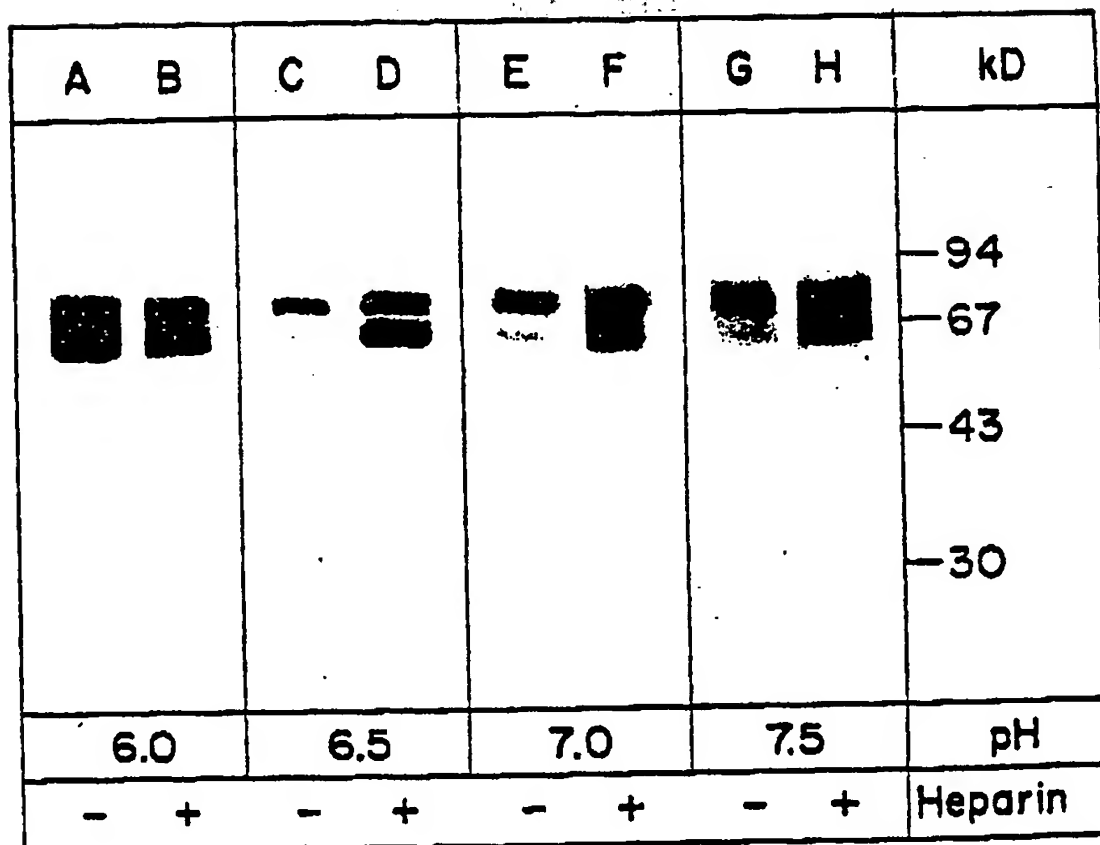


Fig. 6

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Patent Office

## EUROPEAN SEARCH REPORT

Application Number

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DOCUMENTS CONSIDERED TO BE RELEVANT					
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)		
A	EP-A-0 271 810 (TEIJIN LTD) - - -	1	G 01 N 33/50 G 01 N 33/60 G 01 N 33/573 G 01 N 33/68 A 61 K 37/64 C 07 K 15/00		
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The present search report has been drawn up for all claims			TECHNICAL FIELDS SEARCHED (Int. Cl.5)		
Place of search The Hague		Date of completion of search 16 August 91	Examiner CARTAGENA Y ABELLA P		
<table><tr><td><b>CATEGORY OF CITED DOCUMENTS</b> X: particularly relevant if taken alone Y: particularly relevant if combined with another document of the same category A: technological background O: non-written disclosure P: intermediate document T: theory or principle underlying the invention</td><td>E: earlier patent document, but published on, or after the filing date D: document cited in the application L: document cited for other reasons ----- &amp;: member of the same patent family, corresponding document</td></tr></table>				<b>CATEGORY OF CITED DOCUMENTS</b> X: particularly relevant if taken alone Y: particularly relevant if combined with another document of the same category A: technological background O: non-written disclosure P: intermediate document T: theory or principle underlying the invention	E: earlier patent document, but published on, or after the filing date D: document cited in the application L: document cited for other reasons ----- &: member of the same patent family, corresponding document
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